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Phlorotannins From the Brown Algae *Cystophora torulosa* and *Sargassum spinuligerum*

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ABSTRACT Phlorotannins often have various toxic effects against a large number of organisms. From the ethyl acetate fraction of the ethanolic extract of the brown alga *Cystophora torulosa* 33 phlorotannins were obtained. Twenty of them are described in this report: phlorethols and fuhalols, and fucophlorethols and hydroxyfucophlorethols. Seven of them were isolated for the first time. New phlorotannins bearing additional hydroxy groups belong to the hydroxyfucophlorethols. NMR- and MS-data were used for structural elucidation. Several of the substances described for *C. torulosa* occur in *Sargassum spinuligerum* as well. *Nat. Toxins 5:58–63, 1997.*

Key Words: *Cystophora torulosa; Sargassum spinuligerum;* phaeophyceae; phlorotannins; phlorethols; fuhalols; fucophlorethols; hydroxyfucophlorethols; structural elucidation

INTRODUCTION

Phlorotannins are polymers occurring in many brown algae; they all contain phloroglucinol as their monomeric component. They are toxic to a variety of marine animals: hydroids, various worms, pycnogonids, and copepods, as well as larvae. They inhibit fertilization in echinoderms, settlement, and metamorphosis [Ragan and Glombitza, 1986]. Phlorotannins containing extracts are toxic to mice [Fusetani and Hashimoto, 1982]. They inhibit numerous enzymes such as α-amylase, lipase, and trypsin [Barwell et al., 1989], and some are effective antiplasmin inhibitors [Fukuyama et al., 1990]. The brown alga *Cystophora torulosa* contains such phenolic compounds. It is a typical representative of Australasian algae. It grows on the rocky coasts of New Zealand in the littoral and upper part of the sublittoral [Lindauer et al., 1961]. Twenty of the 33 isolated representatives of this class of substances from *C. torulosa* are described in this report. They belong to different groups of phlorotannins which differ from each other in the linkage of their individual phloroglucinol units. Whereas phlorethols and fuhalols are only connected by phenylether bridges, the fucophlorethols have biaryl linkages as well. In every fucophlorethol mentioned in this article, the biaryl element is substituted by a single phloroglucinol element or a chain of phloroglucinol elements, with an ether bridge only on one of the biphenyl elements.

Because the free phlorotannins are sensitive against oxidation, and in order to increase their lipophilic properties, they were isolated as acetates.

EXPERIMENTAL METHODS

Plant Materials

Cystophora torulosa (R. Brown) I. Ag., collected at Whangaparoa, New Zealand, and *Sargasssum spinuligerum* Sonder from Auckland harbour, New Zealand, were frozen as soon as possible and transported frozen to Germany. Voucher specimens are deposited at the herbarium of the University of Auckland.

Mass spectra

Low resolution EIMS data were collected on a Kratos, Manchester MS 50 spectrometer, ion source 200–300°C, ionization energy 70 eV; FABMS data were collected on a Kratos concept 1 with Xe gun and a matrix of 3-nitro-benzylalcohol in the positive mode.

^{1}H-NMR (300 MHz) and ^{13}C NMR (75 MHz)

NMR data were collected on a Varian XL-300 (Bremen, Germany) using solvents as internal standards. The H NMR spectra were recorded both in $CDCl_3$ and d_6-Me_2CO; the ^{13}C NMR spectra in $CDCl_3$. For some signals, the chemical shifts were estimated to the approximated third decimal place. This is to distinguish between signals of very close value but which could nevertheless be clearly differentiated by visual inspection of the spectra.

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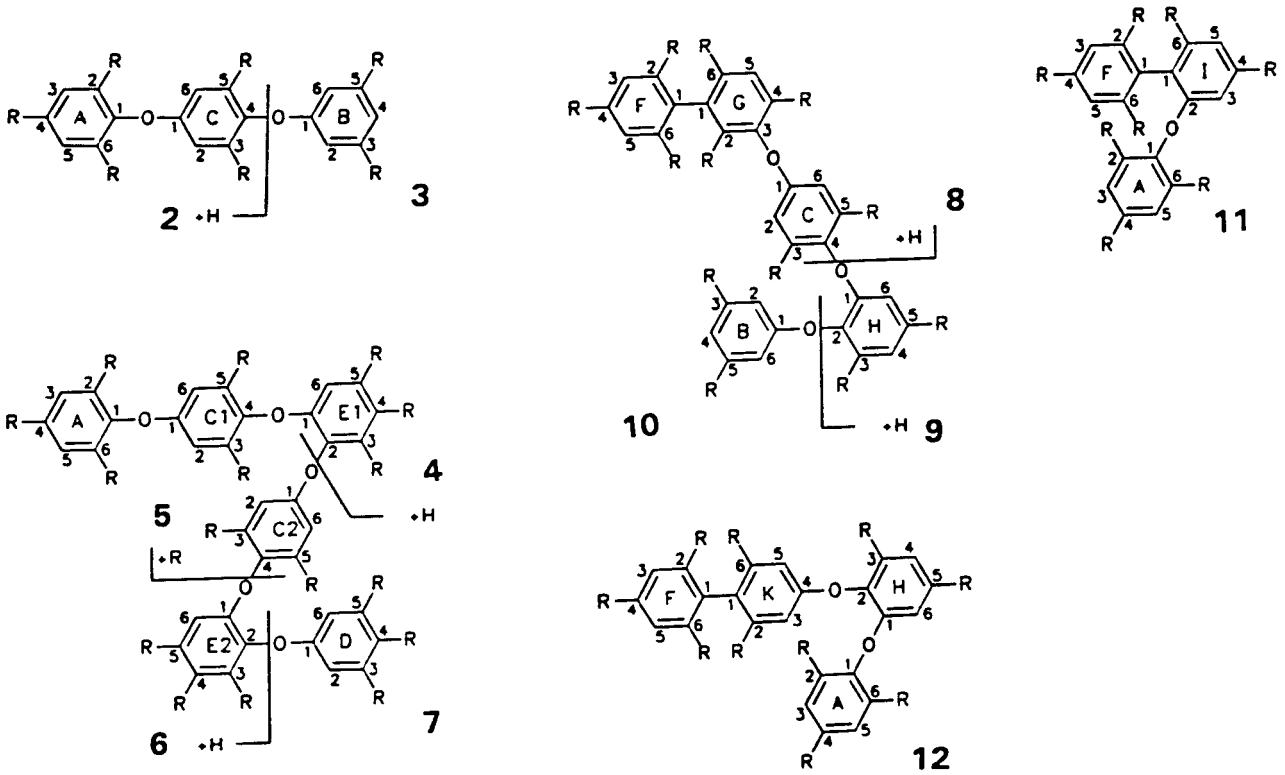

Fig. 1. Compounds **2–12**. Letters in rings are only valid for largest respective compounds. R = OAc.

Extraction and Isolation

Deep-frozen and crushed algae (9 kg) were extracted according to the published procedure [Glombitza et al., 1978]. The free phenols obtained were acetylated with acetanhydride/pyridine for 12 hr. The high molecular mass phlorotannin acetates dissolved in $CHCl_3$ were precipitated by a mixture of petrol/ether, boiling point (bp) 40–60°C (50 + 50). Yield of low molecular mass phlorotannin acetates was 6 g. In 0.5-g quantities, they were preseparated using a low-pressure silica gel column with a stepwise gradient of 200 ml $CHCl_3$/n-hexane (50 + 50), 150 ml $CHCl_3$, 100 ml $CHCl_3/Me_2CO$ (80 + 20), 100 ml $CHCl_3$/MeCO (50 + 50), and 200 ml $CHCl_3$/MeOH (80 + 20). Fine separation was performed using high performance liquid chromatography (HPLC) [pump: Knauer (Berlin, Germany) Fr-30 and 64; gradient programmer: Knauer 50; detector: photodiodes-array-UV/Vis-detector, SPDM6 Shimadzu (Düsseldorf, Germany) at 270/250 nm; column: 250 × 8 mm LiChrosorb Si 60 Merck, (Darmstadt, Germany) 5 µm] with $CHCl_3$/EtOH, $CHCl_3$/EtOH/n-hexane, and $CHCl_3$/EtOH/MeCN mixtures.

Isolated Compounds

Yields correspond to 18 kg frozen algae.

Phloroglucinol triacetate (**1**); 6 mg, identical with Ragan and Glombitza [1986].

Diphlorethol pentaacetate (**2**); 5 mg, identical with Koch and Gregson [1984].

Triphlorethol-A- heptaacetate (**3**); 3 mg, identical with Koch and Gregson [1984].

Trifuhalol-A octaacetate (**4**); 3 mg, identical with Glombitza and Sattler [1973].

Tetrafuhalol-A undecaacetate (**5**); 3 mg, identical with Glombitza et al. [1982].

Pentafuhalol-A tridecaacetate (**6**); 4 mg, identical with Glombitza et al. [1982].

Hexafuhalol-A hexadecaacetate (**7**); 2 mg, identical with Glombitza et al. [1982].

Fucophlorethol-B octaacetate (**8**); 3 mg, identical with Wegner-Hambloch, [1983].

Fucodiphlorethol-D decaacetate (**9**); 5 mg, identical with Glombitza et al. [1981].

Fucotriphlorethol-B dodecaacetate (**10**); 3 mg, identical with Keusgen and Glombitza [1995].

Fucophlorethol-C octaacetate (**11**); 2 mg, identical with Glombitza and Große-Damhues [1985].

Fucodiphlorethol-E decaacetate (**12**); 1 mg, identical with Craigie et al. [1977].

Hydroxyfucophlorethol-A nonaacetate (**13**): 2,4,6,2′,4′,6′-hexaacetoxy-3-(3,4,5-triacetoxyphenoxy)-biphenyl; 4 mg, EI-MS ketene elimination series: m/z 768 → 390, 666 → 372, 434 → 266, and 334 → 250.

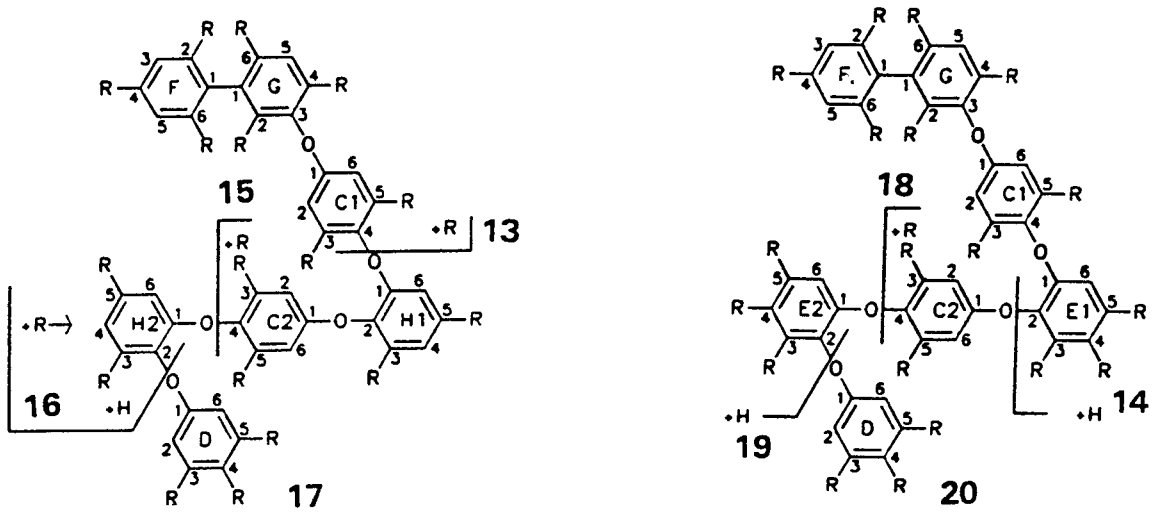

Fig. 2. Compounds **13–20**. Letters in rings are only valid for largest respective compound. R = OAc.

Hydroxyfucodiphlorethol-A undecaacetate (**14**); 4 mg, identical with Glombitza and Li [1991].

Hydroxyfucotriphlorethol-A tridecaacetate (**15**): 2,6,3′,5′-tetraacetoxy-2′-(3,4,5-triacetoxyphenoxy-4-(2,4,6,2′,4′,6′-hexaacetoxybiphenylyl-3-oxy)-diphenylether; 1 mg, EI-MS-ketene elimination series: m/z 1142 → 638, 1082 → 914, 918 → 498, 934 → 514, 710 → 374, 726 → 390, 502 → 250, and 518 → 266; FAB-MS: m/z $[M + K]^+$ 1224, $[M + Na]^+$ 1207; ketene elimination series: m/z 1185 → 639.

Hydroxyfucotetraphlorethol-A pentadecaacetate (**16**): 3,5-diacetoxy-1-[2,6-diacetoxy-4-(2,4,6,2′,4′,6′-hexaacetoxybiphenylyl-3-oxy) [phenoxy]-2-[3,5-diacetoxy-4-(3,4,5-triacetoxyphenoxy)-phenoxy]benzene; 1 mg, EI-MS-ketene elimination series: m/z 1056 → 762, 1122 → 870, 1126 → 622, 1142 → 638, 918 → 498, 934 → 514, 710 → 374, 726 → 390, 502 → 250, and 518 → 266; FAB-MS: m/z $[M + K]^+$ 1431, $[M + Na]^+$ 1415; ketene elimination series: m/z 1393 → 805.

Hydroxyfucopentaphlorethol-A heptadecaacetate (**17**): 4,6,3′,5′-tetraacetoxy-2-[2,6-diacetoxy-4-(2,4,6,2′,4′,6′-hexaacetoxybiphenylyl-3-oxy)phenoxy]-4′-[3,5-diacetoxy-2-(3,4,5- triacetoxyphenoxy)phenoxy]diphenylether; <1 mg, FAB-MS: m/z, $[M + K]^+$ 1639, $[M + Na]^+$ 1623; ketene elimination series: m/z 1601 → 1307.

Dihydroxyfucotriphlorethol-A tetradecaacetate (**18**): 2,6,3′,4′,5′-pentaacetoxy-2′-(3,4,5-triacetoxyphenoxy)-4-(2,4,6,2′,4′,6′- hexaacetoxybiphenylyl-3-oxy)-diphenylether; 3 mg, EI-MS-ketene elimination series: m/z 1158 → 654, 1198 → 720, 892 → 514, 950 → 530, 626 → 374, 684 → 390, 376 → 250, and 476 → 266; FAB-MS: m/z $[M + K]^+$ 1281, $[M + Na]^+$ 1265; ketene elimination series: m/z 1243 → 949.

Dihydroxyfucotetraphlorethol-A hexadecaacetate (**19**): 3,4,5-triacetoxy-1-[2,6-diacetoxy-4-(2,4,6,2′,4′,6′-hexaacetoxybiphenylyl- 3-oxy)phenoxy]-2-[3,5-diacetoxy-4-(3,4,5-triacetoxyphenoxy)phenoxy]benzene; 6 mg, EI-MS-ketene elimination series: m/z 862 → 778, 932 → 638, 864 → 654, 934 → 514, 656 → 530, 626 → 374, 684 → 390, 418 → 250, and 434 → 266; FAB-MS: m/z $[M + K]^+$ (1489), $[M + Na]^+$ 1473; ketene elimination series: m/z 1451 → 905.

TABLE I. ^{13}C-NMR Spectral Data of Compounds 13, 14, and 19*

		Chemical shift			
		Measured			Calculated
Ring type	C-No.	**13**	**14**	**19**	[14]
Ring F	C-4	150.7	150.7	150.7	
	C-2,6	149.2	149.1	149.2	
	C-1	114.9	114.9	114.9	
	C-3,5	113.8	113.8	113.8	
Ring G	C-6	145.0	145.2	145.1	144.2
	C-2	143.0	143.1	143.2	142.9
	C-4	142.7	(−)	142.8	141.6
	C-3	137.0	(−)	137.0	137.8
	C-1	118.2	(−)	118.2	116.5
	C-5	116.1	116.1	116.2	114.8
				C1/C2	
Ring C	C-1		153.4	153.4/154.1	153.1
	C-3,5		143.7	143.7	140.0
	C-4		(−)	134.3 (7)/134.4 (4)	134.7
	C-2,6		109.8	109.5	108.3
Ring E	C-1			147.7	147.5
	C-5			140.1	139.2
	C-3			136.9	136.6
	C-2			134.7	134.4
	C-4			131.4	131.2
	C-6			109.6	109.0
Ring D	C-1	154.2	154.2	154.6	155.2
	C-3,5	143.7	143.7	143.9	144.3
	C-4	130.4	130.3	130.2	130.2
	C-2,6	109.2	109.0	109.0	108.0

*Three hundred MHz, δ in ppm, in $CDCl_3$. (−), signal not detectable, not enough substance.

TABLE II. Correlation of ^{1}HNMR Data of Compounds 13 and 15–17*

	Chemical shift measured			
Ring type	**13** ($CDCl_3/d_6$-Me_2CO)	**15** ($CDCl_3/d_6$-Me_2CO)	**16** ($CDCl_3/d_6$-Me_2CO)	**17** ($CDCl_3/d_6$-Me_2CO)
Ring F				
C-3,5	7.002/7.039	6.999/7.043	6.999/7.043	7.001/7.043
C-4 (Ac)	2.294/2.281	2.294/2.283	2.295/2.284	2.298/2.284
C-2.6 (Ac)	2.056/2.001	2.048/2.002	2.073/2.007	2.048/2.008
Ring G				
C-5	7.111/7.221	7.113/7.235	7.105/7.233	7.110/7.236
C-6 (Ac)	2.068/2.083	2.120/2.125	2.117/2.140	2.111/2.125
C-4 (Ac)	2.023/1.978	2.021/1.979	2.025/1.982	2.025/1.982
C-2 (Ac)	1.905/1.838	1.912/1.847	1.912/1.848	1.912/1.849
Ring C1				
C-2,6		6.673/6.750	6.672/6.785	6.674/6.772
C-3,5 (Ac)		2.014/2.036	2.038/2.064	2.007/2.078
Ring C2				
C-2,6			6.679/6.785	6.652/6.741
C-3,5 (Ac)			2.049/2.052	2.021/2.060
Ring H1				
C-4		6.743/6.865d	6.743/6.872d	6.728/6.848d
C-6		6.500/6.481d	6.475/6.453d	6.475/6.477d
C-5 (Ac)		2.226/2.209d	2.229/2.211d	2.207/2.187d
C-3 (Ac)		2.152/2.138d	2.195/2.166d	2.159/2.139d
Ring H2				
C-4				6.736/6.870d
C-6				6.531/6.532d
C-5 (Ac)				2.224/2.208d
C-3 (Ac)				2.186/2.157d
Ring D				
C-2,6	6.722/6.760	6.692/6.780	6.698/6.728	6.691/6.785
C-4 (Ac)	2.258/2.256	2.251/2.250	2.255/2.250	2.250/2.248
C-3,5 (Ac)	2.240/2.238	2.240/2.230	2.229/2.218	2.239/2.229

*Three hundred MHz, δ in ppm. d, coupling constant J = 2.9/2.7 Hz. Last decimal place shows only a tendency.

Trihydroxyfucopentaphlorethol-A nonadecaacetate (**20**): 4,5,6,3′,5′-pentaacetoxy-2-[2,6-diacetoxy-4-(2,4,6,2′,4′,6′-hexaacetoxybiphenylyl-3-oxy)phenoxy]-4′-[3,5-diacetoxy-2-(3,4,5- triacetoxyphenoxy)phenoxy]diphenylether; 2 mg, FAB-MS: m/z $[M + K]^+$ 1755, $[M + Na]^+$ 1739; ketene elimination series: m/z 1717 → 1255.

RESULTS AND DISCUSSION

The native phlorotannins were extracted from the deep-frozen and crushed algae using ethanol (end concentration 50–60%). To remove interfering lipophilic accompanying substances, the residue obtained after gentle evaporation was extracted with petrol and chloroform. Subsequently, the phenols were transferred to ethyl acetate. After removal of the solvent, the phenols were acetylated immediately with a mixture of acetic anhydride and pyridine. With an ether/petrol mixture, the high molecular mass phlorotannin acetate fraction was separated from that of the low molecular mass fraction by precipitation from $CHCl_3$ solution. The low molecular mass phlorotannin acetates were partially separated using a low-pressure silica gel column. Final separation was carried out by high performance liquid chromatography (HPLC) on silica gel 60 columns using chloroform/ethanol or chloroform/ethanol/n-hexane or chloroform/ethanol/methyl cyanide mixtures. The structures were elucidated using nuclear magnetic resonance (NMR) and mass spectrometry (MS).

The following 13 compounds already isolated from other algae were isolated from *C. torulosa*, and their structures were elucidated by comparison spectra: phloroglucinol triacetate (**1**) [Ragan and Glombitza, 1986]; phlorethols (Fig. 1): diphlorethol pentaacetate (**2**) [Koch and Gregson, 1984], triphlorethol-A heptaacetate (**3**) [Koch and Gregson, 1984] (for phlorethols from *Sargassum spinuligerum* see Keusgen and Glombitza [1995]); fuhalols (Fig. 1): trifuhalol-A octaacetate (**4**) [Glombitza and Sattler, 1973], tetrafuhalol-A undecaacetate (**5**) [Glombitza et al., 1982], pentafuhalol-A tridecaacetate (**6**) [Glombitza et al., 1982], hexafuhalol-A hexadecaacetate (**7**) [Glombitza et al., 1982] (for fuhalols from *S. spinuligerum* see Keusgen and Glom-

bitza [1995] and Glombitza and Keusgen [1995]); fucophlorethols: fucophlorethol-B octaacetate (**8**) [Wiedenfeld, 1977], fucodiphlorethol-D decaacetate (**9**) [Glombitza et al., 1981], fucotriphlorethol-B dodecaacetate (**10**) [Glombitza and Keusgen, 1995], fucophlorethol-C octaacetate (**11**) [Glombitza and Große-Damhues, 1985], fucodiphlorethol-E decaacetate (**12**) [Craigie et al., 1977], and hydroxyfucodiphlorethol-A (**14**) undecaacetate [Glombitza and Li, 1991] (Figs. 1 and 2) (for fucophlorethols from *S. spinuligerum* see also Keusgen et al. [1997]).

Substance **14** is a fucophlorethol with an additional acetoxy group. Hitherto it was the only hydroxyfucophlorethol isolated, and it was found first in *Carpophyllum maschalocarpum* [Glombitza and Li, 1991]. In this paper we report on the isolation of this substance from both *C. torulosa* and *S. spinuligerum.*

This paper describes further representatives of this phlorotannin group isolated from *C. torulosa* or from *S. spinuligerum* (substances **13** and **18**) at the same time. (Fig. 2). This group is characterized by a 2,4,6,2′,4′,6′-hexaacetoxybiphenyl on one end of the molecule (ring F–G), which is linked at C-3 by ether bridges with additional phloroglucinol units. Each terminal phloroglucinol element is substituted by an additional acetoxy group, resulting in a 3,4,5-triacetoxyphenoxyl (ring D). The smallest compound isolated was hydroxyfucophlorethol-A nonaacetate (substance **13**). The electron-impact mass spectrum (EI-MS) shows an μ^+ at mass per charge (m/z) 768. From this, nine ketenes split off which are derived from the acetoxy groups. The simultaneous appearance of a ketene elimination series from m/z 666–372 is traced back to the formation of a furanoid ring between ring F and ring G and is typical for two phloroglucinols linked by a biaryl bond (the next higher homologue is substance **14**).

The higher homologues have either only one additional acetoxy group on the last phloroglucinol ring (substances **15**–**17**), or they have additional acetoxy groups on the rings labelled E1 and E2 (substances **18**–**20**).

The molecular ions in fast atom bombardement mass spectrometry (FAB-MS) (1184, 1392, and 1600) of substances **15**–**17** are at the beginning of a ketene elimination series, in which each ends after loss of 13, 15, or 17 ketenes at the ions of the free five-, six-, or seven-ringed homologues.

The masses determined for substances **18** and **19** are 58 atomic mass units (a.m.u.) higher than those of substances **15** and **16** because they possess an additional acetoxy group. Compound **20** exhibits 116 a.m.u. more than substance **17** because the ring E occurs twice (instead of H in substance **17**). Accordingly, the ketene elimination series could be identified with 14, 16, and 19 elements. EIMS indicates a furanoid ring formation between ring F and G.

The ^{1}H-NMR-spectra of compounds **13**–**20** (Tables I and II) show great similarity with those of the fucophlorethols **8**–**10**. Due to the additional acetoxy group on ring D, the AB_2-system of ring B at 6.66–6.64 ppm and 6.58–6.54 ppm disappears and is replaced by a singlet for 2 H in the range of 6.72–6.69. Simultaneously, a singlet appears at 2.26–2.25 ppm for 3 H for the additional acetoxy group. The AB-system of ring type H at 6.77–6.74 ppm and 6.55–6.49 ppm present in substances **16** and **17** disappears in substances **18**–**20**. Instead, a singlet for 1 H at 6.70–6.62 ppm appears, along with a singlet for 3 H at 2.26–2.24 ppm forming the rings E1 and E2.

To determine the exact position of ring elements of type C within the molecule, the resonances of the aromatic protons from substances **13**–**17** were graphically brought into correlation with those of substances **8**–**10** and of fucotetraphlorethol-A-tetradecaacetate (six-ringed) as well as of fucopentaphlorethol-A-hexadecaacetate (seven-ringed) [Wegner-Hambloch, 1983] by fixing the signal of the aromatic protons of ring F, usually found between 7.00–6.98 ppm, to exactly 6.98 ppm and relating all other signals to this. Substances **18**–**20** and their comparison spectra **4**–**7** were

TABLE III. Correlation of ^{1}HNMR-data of Compounds 18–20*

	Chemical shift measured		
Ring type	**18** ($CDCl_3$/ d_6-Me_2CO)	**19** ($CDCl_3$/ d_6-Me_2CO)	**20** ($CDCl_3$/ d_6-Me_2CO)
Ring F			
C-3.5	6.999/7.040	7.000/7.041	6.998/7.042
C-4 (Ac)	2.295/2.281	2.290/2.283	2.296/2.282
C-2.6 (Ac)	2.047/2.001	2.060/2.002	2.041/2.001
Ring G			
C-5	7.110/7.230	7.108/7.230	7.106/7.228
C-6 (Ac)	2.118/2.130	2.105/2.132	2.100/2.128
C-4 (Ac)	2.020/1.980	2.020/1.982	2.025/1.980
C-2 (Ac)	1.906/1.842	1.905/1.840	1.903/1.838
Ring C1			
C-2,6	6.662/6.766	6.661/6.732	6.658/6.769
C-3,5 (Ac)	2.030/#	2.040/#	2.025/#
Ring C2			
C-2,6		6.701/6.775	6.679/6.756
C-3,5 (Ac)		2.050/#	2.025/#
Ring E1			
C-6	6.658/6.650	6.625/6.642	6.619/6.638
C-4 (Ac)	2.250/2.248	2.255/2.250	2.244/2.245
C-5 (Ac)	2.220/2.221	2.215/2.200	2.215/2.218
C-3 (Ac)	2.167/2.158	2.210/2.221	2.195/2.187
Ring E2			
C-6			6.698/6.698
C-4 (Ac)			2.256/2.258
C-5 (Ac)			2.200/2.187
C-3 (Ac)			2.171/2.161
Ring D			
C-2,6	6.719/6.771	6.710/6.805	6.719/6.782
C-4 (Ac)	2.255/2.263	2.260/2.271	2.258/2.267
C-3,5 (Ac)	2.241/2.235	2.225/2.218	2.239/2.233

*Three hundred MHz, δ in ppm. (#), signal hidden by solvent. Last decimal place shows only a tendency.

treated analogously. The graphic representation shows the close relationship of substances **8**–**10** with **13**–**17** on one side, and of substances **4**–**7** with **14** and **18**–**20** on the other (diagrams may be requested from the authors).

Sufficient quantities of substances **13**, **14**, and **19** could be isolated to obtain ^{13}C-NMR spectra (Table III). A comparison of the measured chemical shifts displayed good conformity with the values calculated by Wegner-Hambloch and Glombitza [1985].

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